In the Title

Please replace the Title with the following:

A METHOD OF PRODUCING A RECOMBINANT PROTEIN ~~OF INTEREST,~~ AND A PROTEIN PRODUCED BY THE METHOD

In the Specification

Please replace paragraphs [0001] through [0002] with the following:

Related Application

This is a §371 of International Application No. PCT/FR2004/001538, with an international filing date of June 18, 2004 (WO 2004/113539, published December 29, 2004), which is based on French Patent Application No. 03/07411, filed June 19, 2003.

Field of the Invention

~~The~~This invention relates to a ~~novel~~ method ~~for~~of producing large quantities of a protein of interest~~,~~ which can be used directly for structural analyses. The invention also relates to the recombinant protein obtained.

Background

G protein-coupled receptors (GPCRs) constitute a superfamily of membrane proteins characterized by 7 transmembrane domains (TM I to VII) which play an essential role in intercellular communication and the reception of sensory signals ~~[(1)]~~.

Please replace paragraphs [0004] through [0013] with the following:

Expressed at the surface of all the cells of an organism (from yeast to ~~man~~humans), they are activated by a large variety of extracellular messages (peptides, hormones, lipids, odorous molecules, light, nucleotides, nucleosides, taste molecules, etc.). Activation thereof gives rise to an intracellular cascade of signals via G proteins and results in a large number of cellular responses (for example, cell division or shrinkage, neurotransmission).

In general, GPCRs are involved in each physiological function. The importance of these receptors and the fact that their location in the cell is known makes them ideal targets for therapy. ~~And in~~In fact, it may be estimated that almost 50% of medicaments on the market act via ~~the~~ GPCRs.

Many pathologies are the result of GPCR mutations, and their clinical manifestations are well known; mention. Mention may be made, for example, of blindness, nephrogenic diabetes insipidus, hypothyroidism or hyperthyroidism, precocious puberty, obesity [(2)].

The discovery that some chemokine receptors are cofactors of infection by the HIV virus reinforces the idea that GPCRs are involved in a wide range of pathological situations [(3)].

These general considerations clearly demonstrate the need to study the functional architecture of these receptors, so as to better understand the signal transduction process and the dynamics of their interactions with various molecules (ligands or intracellular partners), and to develop new pharmacological and therapeutic tools. However, the study of the functional architecture of GPCRs using "direct" experimental methods (X-ray crystallography, NMR, mass spectrometry) still remains very limited. Just one three-dimensional (3D) structure is currently known, that of bovine rhodopsin [(4)], on account of the very high natural level of expression of this receptor in the retina. Knowledge of their functional architecture is thus currently obtained using a set of methods involving theoretical methods (modeling), physicochemical methods (photolabeling, fluorescence) and biological methods (site-specific mutagenesis, molecular pharmacology, knock-out, etc.).

TheseThose studies are of extreme importance on the industrial and socioeconomic levels, given the potential therapeutic applications.

However, studying the structure and function of the GPCRs is very difficult for various reasons:

- the transmembrane nature of these proteins and their hydrophobicity makes them delicate to handle and usually leads to a loss of functionality and to denaturation following solubilization;

- it remains very difficult to obtain them in their complete primary sequence. Most of the time, they are expressed in truncated form [5];
- they are expressed in very low quantity (0.01% of membrane proteins), which forms an obstacle to purifying them in large quantities;
- their molecular weight is high (greater than 40 kD), and they are characterized by the presence of post-translational modifications (glycosylation, palmitoylation, phosphorylation) and particular structural features (disulfide bridges);
- they are multifunctional proteins having domains with different roles: ligand binding, G protein activation, allosteric sites, zones involved in their regulation/desensitization.

It will be easily understood that the critical step which at present forms a real obstacle is ~~surely~~ that of obtaining GPCRs in amounts compatible with "direct" structural biology approaches.

To date, no strategy has been developed for producing them in large quantity and in a way which can be generalized to all GPCRs, and which furthermore allows simple purification thereof in a functional form. At times, some receptors have been produced in high quantities (mg/l of culture) ~~[~~(6-8~~]~~), but the methods used cannot be applied to most GPCRs.

Within the context of producing a protein in large quantities, GPCRs represent only one example of the difficulties encountered when trying to obtain a large quantity of a protein of interest. ~~The object of the present invention is~~It would therefore be advantageous to provide a method for producing a large quantity of a protein of interest, particularly GPCRs.

Summary of the Invention

This invention relates to a fragment of an alpha-integrin for producing at least one recombinant protein of interest in a cell, with the exception of a mammalian cell.

This invention also relates to a recombinant protein including at least one fragment of the alpha-integrin and at least one membrane protein of interest.

This invention further relates to a nucleotide sequence coding for at least one recombinant protein of interest.

This invention still further relates to a vector including the nucleotide sequence.

This invention also further relates to a cell, with the exception of a mammalian cell, into which the nucleotide sequence or the vector has been introduced.

This invention further yet relates to a method for producing at least one protein of interest including introducing into a cell, with the exception of a mammalian cell, the nucleotide sequence coding for at least one recombinant protein, and placing the cell under conditions which allow expression of the recombinant protein(s) of interest.

Brief Description of the Drawings

Fig. 1 shows a construct corresponding to a vector according to aspects of the invention.

Fig. 2 shows production of the α5-integrin/vasopressin V2 receptor fusion protein according to the method of the invention (left-hand column: molecular weight of the proteins of the marker sample; arrow: position of the α5-integrin/vasopressin V2 receptor recombinant protein, NI: proteins of a non-induced sample, 2h, 3h and 4h: proteins of an induced sample after 2h, 3h and 4h of induction).

Fig. 3 shows the α5-integrin/vasopressin V2 receptor recombinant protein of Fig. 2 after purification and migration on electrophoresis gel (left-hand column: molecular weight of the proteins of the marker sample; arrow: position of the α5-integrin/vasopressin V2 receptor recombinant protein).

Fig. 4 shows the result of purification of the α5-integrin/vasopressin V2 receptor CXCR4 (α5-V2-CXCR4) recombinant fusion protein by means of affinity chromatography.

S6M: supernatant of solubilization in 6M urea buffer, deposited on Ni-NTA agarose resin

FT: sample not held on the resin

W: fraction wash containing 15 mM imidazole

E100: purified fusion eluted in a buffer containing 100 mM imidazole

The arrow indicates the position of the α5-V2-CXCR4 fusion protein.

Detailed Description

~~The inventors~~We have surprisingly demonstrated that the construct of recombinant proteins, particularly membrane proteins, and most particularly GPCRs, comprising at least one fragment of an alpha-integrin and the protein of interest, makes it possible to obtain recombinant proteins capable of being expressed in large quantities. This strategy makes it possible in particular to obtain a production of ~~said~~the proteins in a large quantity in microorganisms, particularly in bacteria. When the recombinant proteins ~~of the invention~~ are produced in bacteria, they accumulate in the inclusion body of the bacterial cytoplasm. It is then necessary to renaturate the proteins of interest ~~so as~~ to obtain them in active form in a quantity compatible with direct analysis of their structure, for example, by X-ray crystallography or nuclear magnetic resonance (NMR). The method ~~of the invention~~ can furthermore permit ~~the~~ production of non-truncated proteins, particularly when it is applied to GPCRs.

Please replace paragraphs [0015] through [0018] with the following:

~~As far as the inventors~~We are not aware, that the alpha subunit of integrins (also referred to as alpha-integrin (α-integrin) ~~in the text~~) has ~~never~~ been used to produce recombinant proteins of interest in cells other than mammalian cells, and to do so in a quantity that is directly compatible

with structural analysis of the protein of interest, this requiring a quantity of ~~said~~the protein which may be as much as several milligrams.

~~The present invention aims to meet this requirement.~~

Thus, one aspect of the invention ~~firstly~~ relates to the use of at least one fragment of an alpha-integrin in the construct of at least one recombinant protein of interest. ~~The invention~~It also relates to the use of at least one fragment of an alpha-integrin for producing at least one recombinant protein of interest.

~~In the present text, the~~The expression "recombinant protein" or "recombinant protein of interest" as used herein relates to the recombinant protein produced according to aspects of the invention. This recombinant protein may in particular comprise the chaining of several (at least two) proteins of interest which are fused, and which may optionally be separated by spacer sequences and/or cleavage sequences.

Please replace paragraphs [0021] through [0022] with the following:

"Fragment of an alpha-integrin" will be understood to mean both the complete amino acid sequence of the alpha-integrin used and also a partial sequence. The sequence of the alpha-integrin which is used may be native or mutated. Preferably, ~~according to the invention,~~ the sequence used is a sequence comprising the N-terminal end of the alpha-integrin used, even more preferably a sequence corresponding to the N-terminal end of the alpha-integrin used.

~~According to one particular embodiment of the invention, the~~The fragment of the alpha-integrin used may comprises at least FG-GAP modules IV to VII and a portion of FG-GAP module III of the alpha-integrin used.

Please replace paragraphs [0024] through [0052] with the following:

~~According to one particular embodiment of the invention, use issue~~Use may be made of a fragment of 287 amino acids, corresponding to the part of the N-terminal end of alpha-5-integrin which extends between positions 231 and 517, according to the numbering which takes account of the presence of the signal peptide. If account is not taken of the signal peptide, the fragment which can be used ~~in the invention~~ extends from position 190 (G residue) to 476 (G residue) of alpha-5-integrin.

When use is made of other alpha-integrins, the fragments which can be used ~~according to the invention~~ are the fragments homologous to the fragments defined above. For example, in the case of αV-integrin, the fragment which can be used ~~according to the invention~~ corresponds to the part of the N-terminal end of αV-integrin which extends from position 211 (G residue) to 495 (G residue) according to the numbering which takes account of the presence of the signal peptide. If account is not taken of the signal peptide, the fragment which can be used ~~in the invention~~ extends from position 181 (G residue) to 465 (G residue) of αV-integrin. In the case of αIIb-integrin, the fragment which can be used ~~according to the invention~~ corresponds to the part of the N-terminal end of αIIb-integrin which extends from position 224 (G residue) to 508 (Q residue) according to the numbering which takes account of the presence of the signal peptide. If account is not taken of the signal peptide, the fragment which can be used ~~in the invention~~ extends from position 193 (G residue) to 477 (Q residue) of αIIb-integrin.

~~According to one particular embodiment of the invention, the~~The fragment of the alpha-integrin used may comprises at least one amino acid sequence selected from the sequences SEQ ID No. 1 (fragment of human α5-integrin), SEQ ID No. 2 (fragment of human V-integrin) and SEQ ID No. 3 (fragment of human αIIb-integrin) in the appended sequence listing.

~~According to another particular embodiment of the invention, the~~The alpha-integrin fragment used may comprises at least one amino acid sequence encoded by one of the nucleotide sequences selected from the sequences SEQ ID No. 4 (fragment of human α5-integrin), SEQ ID No. 5 (fragment of human V-integrin) and SEQ ID No. 6 (fragment of human αIIb-integrin) in the appended sequence listing.

~~According to yet another particular embodiment of the invention, it~~It is possible that the fragment of alpha-integrin is used in the construct of several (at least two) recombinant proteins of interest. In this case, the recombinant proteins will be fused during translation. This may prove necessary in the case of a protein of interest in respect of which the construct ~~according to the invention~~ does not allow its direct production (refractory protein). It is then necessary to couple in tandem the sequence of ~~said~~the refractory protein to a recombinant protein of interest which the constructs ~~according to the invention~~ make it possible to produce. Thus, ~~according to this particular embodiment of the invention,~~ the construct ~~according to the invention will~~may comprise at least one DNA fragment encoding at least one fragment of an alpha-integrin, then at least one DNA encoding at least a first recombinant protein of interest and at least one DNA encoding at least a second recombinant protein of interest. ~~According to this particular embodiment of the invention, the~~The DNA encoding the second protein of interest ~~will~~may be inserted in the construct in phase downstream of the DNA sequence encoding the first protein of interest. This ~~particular embodiment~~ can be combined with any one of the ~~particular embodiments~~aspects described above.

Preferably, ~~according to the invention,~~ the alpha-integrin fragment is located in the recombinant protein of interest ~~prepared according to the invention~~, upstream of the sequence of the protein of interest (or proteins of interest) to be produced, that is to say at the N-terminal end of the

recombinant protein of interest (or recombinant proteins of interest) which are to be constructed and/or produced.

~~The~~Aspects of the invention also relates to a recombinant protein, ~~characterized in that it comprises~~comprising, fused together, at least one fragment of an alpha-integrin as defined above and at least one protein of interest.

The protein(s) of interest, which forms (form) part of the recombinant protein ~~of the invention~~, may be any protein which it is desired to produce, particularly a membrane protein, more particularly a G protein-coupled receptor (GPCRs). By way of example of the latter, mention may be made of vasopressin and oxytocin receptors (V1a, V2, OTR), leukotriene receptors (BLT1, BLT2, CysLT1, CysLT2), adrenergic receptors (beta-3), cannabinoid receptors (CB1), chemokine receptors (CCR5, CXCR4), the angiotensin II AT1 receptor, the bradykinin B2 receptor.

The recombinant protein ~~of the invention~~ (regardless of the embodiment of the invention) may further~~more~~ comprise any amino acid sequence which makes it possible to purify ~~said~~the protein in a simple manner. Thus, ~~according to one particular embodiment of the invention,~~ the recombinant protein may comprise a sequence of 6 histidine residues (6xHIS tag; SEQ ID NO: 12). This 6xHIS (SEQ ID NO: 12) tag may be incorporated in the sequence of the protein with a view to its purification on a Ni-NTA (nickel-nitrilotriacetic acid) agarose column. Preferably, this sequence is at the C-terminal end of the recombinant protein ~~of the invention~~. When the recombinant protein ~~according to the invention~~ consists of at least two fused proteins, the 6xHIS tag (SEQ ID NO: 12) is preferably located downstream of the last of the proteins of interest which it is desired to produce.

The sequence encoding the recombinant protein may further~~more~~ comprise at least one sequence encoding at least one endoprotease cleavage site.

Advantageously, the sequence coding for the last residues of the integrin may be mutated to form an endoprotease cleavage site (factor Xa, thrombin), which, following expression and purification of the recombinant protein, will make it possible to separate the protein of interest from its fusion partner. ~~According to one particular embodiment of the invention, the~~The L residue (position 285) may be modified by mutation into an I residue, the E and G residues (positions 286 and 287) being preserved. An additional R residue may be introduced by mutagenesis. The chain thus formed (IEGR: SEQ ID NO: 9) corresponds to the factor Xa cleavage site which cuts the protein after the R residue.

~~In another embodiment, the~~The factor Xa cleavage site can be transformed into a thrombin cleavage site. To do this, the I, E and G residues can be replaced by L, V and P residues. The R residue is preserved ~~so as~~ to obtain the chain LVPR (SEQ ID NO: 10). Since the integrin fragment has been incorporated into the vector at the 3' end by a BamHI site (sequence ggatcc), there is thus obtained the sequence ggatcc coding for two residues G and S just after LVPR (SEQ ID NO: 10). The LVPRGS (SEQ ID NO: 11) chain forms the thrombin cleavage site, which cuts the protein after the R residue.

It will thus be understood that~~, in a more elaborate embodiment,~~ the recombinant protein ~~of the invention~~may comprises, from its N-terminal end to its C-terminal end, the alpha-integrin fragment comprising the endoprotease cleavage site, the protein(s) of interest and the 6xHIS tag (SEQ ID NO: 12).

~~In one particular embodiment, the~~The recombinant protein ~~of the invention~~may comprises, from its N-terminal end to its C-terminal end, the alpha-integrin fragment comprising the factor Xa cleavage site, the protein(s) of interest and the 6xHIS tag (SEQ ID NO: 12).

~~In another particular embodiment, the~~The recombinant protein ~~of the invention~~may comprises, from its N-terminal end to its C-terminal end, the alpha-integrin fragment comprising the thrombin cleavage site, the protein(s) of interest and the 6xHIS tag (SEQ ID NO: 12).

It may still be necessary, when the recombinant protein ~~according to the invention~~ comprises more than one protein of interest which are fused together, that ~~said~~the proteins of interest can be separated after synthesis, for example, before purification. Thus, it is possible to insert, between the different DNA sequences encoding the different proteins of interest, at least one DNA sequence encoding an endoprotease cleavage site. It is possible for cleavage sites for different endonucleases to be inserted into the same recombinant protein.

It may be necessary to make the cleavage of the recombinant protein even more effective. In this respect, it is possible to insert into the construct ~~according to the invention~~ a sequence encoding a peptide sequence which serves as a spacer arm, preferably located upstream of the endoprotease cleavage site.

Thus, ~~according to one particular embodiment of the invention,~~ the recombinant protein may further~~more~~ comprises a peptide sequence serving as a spacer arm, preferably located upstream of the endoprotease cleavage site.

Therefore, ~~in an even more elaborate embodiment,~~ the recombinant protein ~~of the invention~~may comprises, from its N-terminal end to its C-terminal end, the alpha-integrin fragment comprising a spacer arm and the endoprotease cleavage site, the protein(s) of interest and the 6xHIS tag (SEQ ID NO: 12).

~~In one particular embodiment, the~~The recombinant protein ~~of the invention~~may comprises, from its N-terminal end to its C-terminal end, the alpha-integrin fragment comprising a spacer arm, the factor Xa cleavage site, the protein(s) of interest and the 6xHIS tag (SEQ ID NO: 12).

~~In another particular embodiment, the~~The recombinant protein ~~of the invention~~may also comprises, from its N-terminal end to its C-terminal end, the alpha-integrin fragment comprising a spacer arm, the thrombin cleavage site, the protein(s) of interest and the 6xHIS tag (SEQ ID NO: 12).

~~According to the invention, the~~The sequence encoding a peptide sequence which serves as a spacer arm may be any sequence known ~~to the person skilled~~ in the art which allows a sufficient spacing between the endoprotease cleavage site and the protein(s) of interest for the cleavage of the recombinant protein to be effective.

Preferably, ~~said~~the sequence encoding a peptide sequence serving as a spacer arm is the following sequence SEQ ID No. 7: 5' GACCCGGGTGGTGGTGGTGGTGGTGGTGGT 3' encoding the following peptide sequence SEQ ID No. 8: DPGGGGGGGG.

Thus, ~~in a most elaborate embodiment,~~ the recombinant protein ~~of the invention~~may comprises, from its N-terminal end to its C-terminal end, the alpha-integrin fragment comprising a spacer arm and the endoprotease cleavage site, the protein(s) of interest, separated by one or more endoprotease cleavage sites (for example, factor Xa or thrombin cleavage sites) and the 6xHIS tag (SEQ ID NO: 12).

~~The~~Aspects of the invention also relates to the use of at least one fragment of a nucleotide sequence coding for at least one fragment of an alpha-integrin as defined above, in the construct of a nucleotide sequence coding for a recombinant protein of interest as defined above.

~~The~~Aspects of the invention ~~also~~further relates to the use of at least one fragment of a nucleotide sequence coding for at least one fragment of an alpha-integrin as defined above, for producing a recombinant protein of interest as defined above.

~~The~~Aspects of the invention ~~also~~still further relates to a nucleotide sequence coding for a recombinant protein of interest comprising at least one fragment of a nucleotide sequence coding for at least one fragment of an alpha-integrin, as defined above, and a nucleotide sequence coding for at least one protein of interest, as defined above.

Preferably, the nucleotide sequence coding for at least one fragment of an alpha-integrin which can be used ~~according to the invention~~ or is included in the nucleotide sequence coding for a recombinant protein of interest ~~according to the invention~~and may be selected from the nucleotide sequences SEQ ID No. 4, SEQ ID No. 5 and SEQ ID No. 6 in the appended sequence listing.

~~The~~Aspects of the invention ~~also~~yet further relates to a vector comprising a nucleotide sequence coding for a recombinant protein of interest, as defined above, comprising at least one fragment of a nucleotide sequence coding for at least one fragment of an alpha-integrin and a nucleotide sequence coding for at least one protein of interest. The vector may be a eukaryotic vector such as a plasmid or a virus. The vector may also be any prokaryotic vector such as a plasmid or a phage.

Please replace paragraphs [0055] through [0067] with the following:

~~The~~Aspects of the invention also relates to a cell, into which a nucleotide sequence coding for a recombinant protein of interest, as defined above, has been introduced. ~~According to one particular embodiment of the invention, the~~The sequence ~~has been~~may be introduced in the form of a vector as defined above.

"Cell" ~~in this case~~ will be understood to mean both a eukaryotic cell and a prokaryotic cell, particularly a bacterium. Any bacterium capable of allowing the expression of a protein from a nucleotide sequence may be used ~~according to the invention~~. By way of example, mention may be

made of all bacteria which derive from BL21, BL21 star, Rosetta, BLR, Origami, Tuner, Novablue, all commercially available.

The ~~invention also relates to a~~ method ~~for producing~~produces at least one protein of interest, characterized in that, in a first step, there is introduced into a cell a nucleotide sequence coding for a recombinant protein of interest, as defined above, and in that, in a second step, the cell is placed under conditions sufficient for allowing the expression of the recombinant protein of interest.

The method ~~of the invention~~ may further~~more~~ comprise an additional step during which the recombinant protein of interest may be cut by the action of an endoprotease (factor Xa, thrombin, for example), at the site created in the last residues of the integrin ~~so as~~ to separate the protein of interest from its fusion partner.

The method ~~of the invention~~ may also comprise an additional step during which the recombinant protein of interest, or the protein(s) of interest separated from its (their) fusion partner(s), may be purified.

~~According to the method of the invention, the~~The nucleotide sequence coding for a recombinant protein of interest may be introduced into the cell by any known method. By way of example of methods which can be used, it is possible to mention, in respect of prokaryotic cells, heat shock or electroporation. In respect of eukaryotic cells, mention may be made of electroporation, the calcium phosphate precipitate method, the use of cationic polymers such as DEAE-dextran, or any method using cationic liposomes or activated dendrimers. It is also possible to use retroviruses to carry out gene transfer, and also techniques using microprojectiles to deliver DNA to target cells.

Likewise, any sufficient condition known ~~to the person skilled~~ in the art which allows the expression of the recombinant protein of interest can be used according to the method ~~of the invention~~.

Finally, any method of purifying the protein(s) which is known ~~to the person skilled~~ in the art can be used ~~according to the method of the invention~~. By way of example, mention may be made of the methods of affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography or filtration using a molecular sieve.

In particular, when the recombinant protein of interest comprises the 6xHIS tag (SEQ ID NO: 12), purification on a nickel-nitrilotriacetic acid (Ni-NTA) agarose column represents one method of purification which is particularly satisfactory within the context of the method of the invention.

The techniques which can be used ~~according to the invention~~ are known ~~to the person skilled~~ in the art. The latter can refer to the numerous manuals which are available, and in particular to "Molecular Cloning, a laboratory manual. 2nd edition, Sambrook, Fritsch, Maniatis eds., CSH laboratory press, (1989)".

~~Besides the above provisions, the invention also comprises other provisions which will emerge from the following description which refers to examples of embodiments of the invention and also to the appended drawings, in which:~~

~~Fig. 1 shows a construct corresponding to a vector according to the invention.~~

~~Fig. 2 shows the production of the α5 integrin/vasopressin V2 receptor fusion protein according to the method of the invention (left-hand column: molecular weight of the proteins of the marker sample; arrow: position of the α5 integrin/vasopressin V2 receptor recombinant protein, NI: proteins of a non-induced sample, 2h, 3h and 4h: proteins of an induced sample after 2h, 3h and 4h of induction).~~

~~Fig. 3 shows the α5 integrin/vasopressin V2 receptor recombinant protein of Fig. 2 after purification and migration on electrophoresis gel (left-hand column: molecular weight of the~~

~~proteins of the marker sample; arrow: position of the α5 integrin/vasopressin V2 receptor recombinant protein).~~

~~Fig. 4 shows the result of purification of the α5 integrin/vasopressin V2 receptor CXCR4 (α5 V2 CXCR4) recombinant fusion protein by means of affinity chromatography.~~

~~S6M: supernatant of solubilization in 6M urea buffer, deposited on Ni NTA agarose resin~~

~~FT: sample not held on the resin~~

~~W: fraction wash containing 15 mM imidazole~~

~~E100: purified fusion eluted in a buffer containing 100 mM imidazole~~

~~The arrow indicates the position of the α5 V2 CXCR4 fusion protein.~~

The following examples illustrate selected aspects of the invention and do not limit it in any way.

Please replace paragraph [0069] with the following:

The vector directly provides the sequence coding for the 6xHIS tag (SEQ ID NO: 12) which will be located at the C-terminal end of the recombinant protein of interest. An EcoRI site is located in the vector at the N-terminal end of the tag site. Thus, the complementary DNA coding for the protein of interest is inserted between the BamHI site marking the C-terminal end of the complementary DNA fragment of the α5-integrin and the EcoRI site located at the N-terminal end of the 6xHIS tag (SEQ ID NO: 12).

Please replace paragraph [0072] with the following:

Recognition sites for the restriction enzymes BamHI and EcoRI are added on either side of the complementary DNA sequence of the human vasopressin V2 receptor. This is done using the conventional PCR technique. The complementary DNA of the human vasopressin V2 receptor is

amplified from the vector pRK5-V2 (Cotte et al., J. BIOL. Chem. 273, 29462-29468, 1998) with the aid of two primer oligonucleotides which make it possible to insert the desired restriction sites:

sense oligo (allows the incorporation of the BamHI site): 5' ATG GGT CGC GGA TCC ATG CTC ATG GCG TCC ACC ACT TCC 3' (SEQ ID NO: 13)

antisense oligo (allows the incorporation of the EcoRI site): 5' CGA CGG AAT TCT GCG ATG AAG TGT CCT TGG CCA G 3' (SEQ ID NO: 14).

Please replace paragraph [0079] with the following:

Step 34: subcloning of the amplified PCR V2 fragment in the BamHI and EcoRI sites of the vector pET21a of Example 1:

Ligation is carried out by incubating at ambient temperature (20-25°C) for 4 hours in a medium comprising:

- BamHI/EcoRI PCR V2 fragment (100 to 200 ng)	8 µl
- vector pET21a (30 ng) cut by BamHI/EcoRI	3 µl
- 10X ligase buffer (NEB)	2.5 µl
- T4 DNA ligase (NEB)	2 µl
- water	9.5 µl.

Please replace paragraph [0093] with the following:

A colony isolated on LB agar + ampicillin (100 µg/ml) is pricked and cultured in 10 ml of culture medium LB + carbenicillin (100 µg/ml). Culturing is carried out at 37°C, with stirring at 300 rpm. When the optical density of the culture reaches 0.6, culturing is stopped and the culture is kept in the refrigerator (this sample is called the "preculture"). The next day, in a 500 ml Erlenmeyer, 100 ml of culture medium LB + carbenicillin (100 µg/ml) are seeded with 2 ml of preculture and left at 37°C, at 300 rpm, until the optical density of the culture has reached 0.6. 0.1 mM IPTG is then

added to the culture ~~so as~~ to induce expression of the recombinant protein. Culturing is continued for around 3 hours, until an optical density of 2.4 is obtained (stimulation factor of 4).

Please replace paragraph [0102] with the following:

~~In order to solubilize the inclusion bodies and thus the protein of interest, the~~The pellet is taken up by homogenization using a pipette in 5 ml of Tris-HCl 20 mM, pH 8.00, 6M ruea, SDS 0.2% to solubilize the inclusion bodies and thus the protein of interest.

Please replace paragraphs [0105] through [0106] with the following:

The whole is then centrifuged at 15000 rpm for 30 minutes at 4°C. The supernatant contains the protein of interest and constitutes the sample which will be brought into contact with the Ni-NTA (nickel-nitrilotriacetic acid) resin ~~so as~~ to purify the alpha5-V2 fusion by means of affinity chromatography.

3 ml of Superflow Ni-NTA agarose resin (Qiagen, ref. 30430) are equilibrated in Tris-HCl 20 mM, pH 8.00, 6M urea, SDS 0.2%, NaCl 150 mM, imidazole 5 mM. A sufficient amount of NaCl and imidazole are added to the sample containing the protein of interest ~~so as~~ to obtain a final concentration of 150 mM of NaCl and 5 mM of imidazole. The sample and the resin are brought into contact and ~~are~~ left to incubate at 4°C for 16 hours with gentle stirring. The sample/resin mixture is deposited in a plastic column and left. After settling, the "flow-through" fraction is recovered at a low flow rate, for control on electrophoresis gel.

Please replace paragraph [0112] with the following:

The purified sample is dialyzed against a solution of Tris-HCl 20 mM, pH 8.00, 6M urea, NaCl 150 mM ~~so as~~ to remove the SDS and the imidazole. To do this, the sample is placed in a Pierce dialysis cassette (membrane of 10000 MWCO) and dialysis is carried out in a beaker containing one liter of buffer. The dialysis is carried out at 4°C for at least 24 hours.

Please replace paragraph [0115] with the following:

A complementary DNA coding for a protein of interest, in this case the human chemokine receptor CXCR4, is inserted in the vector pET21a(+)-α5V2 described above in Example 2. This DNA must be in phase with that coding for the α5V2 fusion and is positioned between the SacI and HindIII restriction sites for example. The vector directly supplies the sequence coding for the 6xHIS tag (SEQ ID NO: 12) which will thus be located at the C-terminal end of the receptor CXCR4 and will therefore allow its purification in a subsequent step.

Please replace paragraph [0117] with the following:

Recognition sites for the restriction enzymes SacI and HindIII are added on either side of the sequence coding for the human receptor CXCR4 during a conventional PCR reaction. The complementary DNA of this receptor is amplified from the vector pET101/D-TOPO (Invitrogen) in which it is subcloned and from two primer oligonucleotides which make it possible to insert the restriction sites in question.

Sense oligo (incorporation of the SacI site): 5' CGAGCTAAGGC GAGCTC A ATGGAAGGCATTAGCATTTATAC 3' (SEQ ID NO: 15)

Antisense oligo (incorporation of the HindIII site): 5' CGACGGCCC AAGCTT GCTGCTATGAAAGCTGCTGCTTTC 3' (SEQ ID NO: 16).

Please replace paragraphs [0127] through [0129] with the following:

Step 5: transformation of the Rosetta bacteria

Follow ~~to the letter~~ the protocol described in Example 2.

Step 6: expression of the α5V2-CXCR4 fusion

Follow ~~to the letter~~ the protocol described in Example 2, but the LB culture medium is replaced by Hyperbroth medium (Athena Enzyme Systems) and the optimal induction time is 4

hours.

Step 7: purification of the α5V2-CXCR4 fusion. This step is shown in Fig. 4.

Follow ~~to the letter~~ the protocol of Example 4, but, during the step of washing the Ni-NTA agarose resin, a concentration of 15 mM of imidazole instead of 20 mM is used in the wash solution. Elution is carried out to 100 mM as in Example 4.